

Response to Notice of Non-Responsive Amendment

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Applicant(s): Benson et al.

Serial No.: 10/027,277

Confirmation No.: 3061

Filed: December 21, 2001

For: CRYSTALLIZATION AND STRUCTURE DETERMINATION OF GLYCOSYLATED HUMAN BETA
SECRETASE, AN ENZYME IMPLICATED IN ALZHEIMER'S DISEASE

Amendments to the Specification

Please replace the paragraph beginning at page 18, line 4, with the following amended paragraph.

Structure coordinates can be used to aid in obtaining structural information about another crystallized molecule or molecular complex. The method of the invention allows determination of at least a portion of the three-dimensional structure of molecules or molecular complexes which contain one or more structural features that are similar to structural features of human beta secretase. These molecules are referred to herein as "structurally homologous" to human beta secretase. Similar structural features can include, for example, regions of amino acid identity, conserved active site or binding site motifs, and similarly arranged secondary structural elements (e.g., α helices and β sheets). Optionally, structural homology is determined by aligning the residues of the two amino acid sequences to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. Preferably, two amino acid sequences are compared using the Blastp program, version 2.0.9, of the BLAST 2 search algorithm, as described by Tatusova et al., *FEMS Microbiol Lett* 174, 247-50 (1999), and available on the ncbi website with extension [nlm.nih.gov/gorf/bl2.html](http://www.ncbi.nlm.nih.gov/gorf/bl2.html) at <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>. Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and filter on. In the comparison of two amino acid sequences using the BLAST search algorithm, structural similarity is referred to as "identity." Preferably, a structurally homologous molecule is a protein that has an amino acid sequence sharing at least 65% identity with a native or recombinant amino acid sequence of human beta secretase (for example, SEQ ID NO:1). More preferably, a protein that is structurally homologous to human beta secretase includes at least one contiguous stretch of at least 50 amino acids that shares at least 80% amino acid sequence identity with the analogous portion of the native or recombinant human beta secretase (for example, SEQ ID NO:1).

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Methods for generating structural information about the structurally homologous molecule or molecular complex are well-known and include, for example, molecular replacement techniques.

Please replace the paragraph beginning at page 34, line 14 (as amended in the Preliminary Amendment submitted June 11, 2003), with the following amended paragraph.

The 40-80% ammonium sulfate pellet was dissolved in 25 mM Tris-HCl (8.5)/0.5 M NaCl/10 mM imidazole ~~25 mM Tris-HCl (8.5)/0.5 M NaCl/10 mM imidazole~~ (1/10 the original volume) and applied to a 12.5 ml column containing Ni²⁺-NTA Fast Flow resin previously equilibrated in the same buffer. Following sample application, the column was washed with 10 column volumes of loading buffer and then eluted with 25 mM Tris-HCl (8.5)/0.5 M NaCl/50 mM imidazole. The material eluting in 50 mM imidazole was pooled, concentrated approximately 10-fold using a YM 30 membrane (30,000 MWCO), and then dialyzed against 10 mM HEPES-Na (8.0) using 50,000 molecular weight cutoff tubing. For affinity purification, the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine, PNU-292593E, the structure being illustrated in Figure 2) (SEQ ID NO:3) was synthesized and coupled to sulfolink resin (Pierce Chemical Company) as recommended by the manufacture. The dialyzed material from above was adjusted to 0.1 M NaOAc (4.5) by addition of 1/10 volume of 1.0 M NaOAc (4.5) and immediately applied to the PNU-292593E/sulfolink column (6 ml containing 1.0 mg PNU-292593/ml of resin) that had been previously equilibrated in 25 mM NaOAc (4.5). Following sample application, the column was washed with 10 column volumes of 25 mM NaOAc (4.5) and then eluted with 50 mM NaBO₃ (8.5). N-terminal sequence analysis of the affinity purified material revealed an equimolar mixture of pro- and processed human beta-secretase beginning at Thr¹ and Glu²⁵ respectively. The final protein concentration was determined by amino acid analysis assuming a 60 kDa glycoprotein.

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Please replace the paragraph beginning at page 38, line 9, with the following amended paragraph.

Protein Crystallization. Based on observations of the initial screening effort, fresh protein derived from CHO cells was concentrated to 42mg/ml and mixed with a new inhibitor (Figure 1) (~~Figure 1b~~) so that the final concentration of the mix was 40mg/ml beta secretase, and 2mM of the inhibitor shown in Figure 1 (~~Figure 1b~~), in 20mM Hepes pH 7.8, 10% DMSO. This preparation was screened with Hampton Screen 1 (Hampton Research, Laguna Nigel, CA.) and Wizard Screen 1 (Emerald Biostructures, Bainbridge Island, WA) at room temperature (20°C). 500-µL well volumes were used. A 1:1 ratio of protein-compound mix to the well solution was used in a hanging drop format to complete the screen. After 10 days, but less than 18 days later crystals were observed in Wizard Screen 1, Condition No. 45 (20% PEG 3000, 0.1M NaOAc⁻ pH 4.5). Optimization and seeding efforts around this condition provided crystals that grew in 17-20% PEG 3000, 0.1M Na Acetate pH 4.5. Seeding was done utilizing a cat whisker which was touched to a drop containing microcrystals and stepwise diluted by streaking through one row of the optimization tray. Cross-seeding efforts provided crystals of HEK 293 cell derived protein (38mg/ml in 20mM Hepes pH 7.8, 50mM NaCl, 10% DMSO, 2mM of the inhibitor shown in Figure 1 (~~Figure 1b~~)) from CHO cell derived seed stock. Macroseeding by moving small crystals with a loop from the target drop to a fresh drop containing 17-20% PEG3000, 0.1M Na Acetate pH 4.5 also resulted in crystals that doubled or more in size, usually with a shower of microcrystals also. Crystals were obtained in hanging drop or sitting drop methods by seeding. Crystals obtained from streak seeding attempts were frozen in a cryoprotectant solution based on the mother liquor plus 20% Ethylene Glycol. The crystals were then soaked incrementally through 5% intervals of the cryoprotectant in 3 to 5-minute intervals. Crystals have also been grown in the presence of 10% glycerol or 10% ethylene glycol to facilitate stabilization into cryogenic solutions. In these cases, the crystals were soaked incrementally through 5% intervals of the cryoprotectant in 3 to 5-minute intervals. The use of other cryo agents would be apparent to one of skill in the art.

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Please replace the paragraph beginning at page 39, line 24, with the following amended paragraph.

All data collection was carried out at the Advanced Photon Source (Argonne, IL) at beamline 17-ID. The crystals diffracted to 3.2Å using synchrotron radiation. Crystals were of the space group $P3_221$ with cell constants $a=112 \pm 20$ Å, $b=112 \pm 20$ Å, $c=110 \pm 20$ Å, $\alpha=\beta=90^\circ$, $\gamma=120^\circ$. The Matthews coefficient for these crystals assuming that there is one molecules in the asymmetric unit is 3.5Å/Da with 65% solvent. The structure determination (see below) revealed the presence of electron density in the active site appropriate for the inhibitor shown in Figure 1 ~~Figure 1(B)~~.

Please replace the paragraph beginning at page 40, line 4, with the following amended paragraph.

A molecular replacement solution was determined using AMORE (Navaza, Acta Cryst., D50:157-63 (1994); Collaborative Computational Project N4, Acta Cryst. D50:760-3 (1994)) by utilizing a previously published model of human beta secretase, 1FKN, (Hong et al., Science 290:150-53 (2000)) made available from the Protein Data Bank on the world wide web at rcsb.org (www.rcsb.org). Using the 1FKN model, the initial rotation solution gave a single strong peak of 9.7σ with the next strongest peak appearing at 4.0σ . The final determination of the space group ($P321$, $P3_121$, or $P3_221$) was determined experimentally by testing translation searches in each space group. A translation search in the correct space group, $P3_221$, resulted in a correlation coefficient of 55.1 with an R-factor of 39.9% to 4Å resolution.

Please replace the paragraph beginning at page 41, line 9, with the following amended paragraph.

At this point, inspection of the electron density map within the active site revealed electron density that was unaccounted for by the protein model and consistent with the shape of the inhibitor shown in Figure 1 ~~Figure 1B~~ that was present in the crystallization conditions. Model building was done using the program CHAIN (Sack, Journal of Molecular Graphics

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6:224-25 (1988)) and LORE (Finzel, *Meth. Enzymol.* 277:230-42 (1997)). Modest rebuilding of the model into the 3.2Å low resolution map afforded the opportunity for further cycles of refinement giving improvement of the R-factor to 31.6% and a Free R-factor of 35.7% . Finally, the inhibitor was included in the refinement to give the current R-factor of 29.9% and a Free R-factor of 34.9%.

Please replace the paragraph beginning at page 42, line 7, with the following amended paragraph.

All data collection was carried out at the Advanced Photon Source (Argonne, IL) at beamline 17-ID. The crystals diffracted to 2.7Å using synchrotron radiation. Crystals were of the space group P3₂2₁ [[P32₂1]] with cell constants $a=99.4 \pm 35$ Å, $b=99.4 \pm 35$ Å, $c=117 \pm 35$ Å, $\alpha=\beta=90^\circ$, $\gamma=120^\circ$. The Matthews coefficient for these crystals assuming that there is one molecule in the asymmetric unit is 2.9Å/Da with 58% solvent. The structure determination (see below) revealed the presence of electron density in the active site appropriate for the inhibitor shown in Figure 1 ~~Figure 1(B)~~.

Please replace the paragraph beginning at page 42, line 17, with the following amended paragraph.

A molecular replacement solution was determined using AMORE (Navaza 1994; Collaborative Computational Project N4, *Acta Cryst.* D50:760-3 (1994))) by utilizing the structure of human beta secretase produced from CHO cells. Using the CHO beta secretase model, the initial rotation solution gave a strong peak of 10.2σ with the next strongest peak appearing at 8.0σ . The space group was P3₂2₁ [[P32₂1]]. A translation search in the correct space group, P3₂2₁ [[P32₂1]], resulted in a correlation coefficient of 57.8 with an R-factor of 41.5% to 4Å resolution.